REMARKS

Upon entry of this amendment, claims 1, 4, 7-14 and 16-25 will be pending in this application, with claims 14 and 17-20 being withdrawn. Support for the amendment to claim 1 adding the term "isolated" can be found in the specification, for example, at page 9, lines 9-13. Support for the amendment to claim 1 adding the term "interfere with" can be found in the specification, for example, at page 4, lines 1-8. Support for other amendments to claim 1 can be found in the specification, for example, from page 3, line 32 to page 4, line 18; and throughout the Examples. Support for the amendment to claim 16 adding the term "disease" can be found in the specification, for example, at page 4, lines 15-18. Support for the remaining amendments to the claims can be found in the original claims and throughout the specification. Applicants have not raised any issue of new matter.

Applicants have also amended the specification to replace internet citations with alternative means for describing where the information can be found. Additionally, Applicants have amended the abstract as requested by the Examiner. It is believed that these amendments do not add any new matter and their entry is respectfully requested.

The specification is in the proper format. The present claims define patentable subject matter and are believed to be in condition for allowance.

Specification Objections

The Examiner has made objections to the specification based upon its inclusion of browser-executable code and inclusion of "legal phraseology such as said" in the abstract. Solely to expedite prosecution and not in acquiescence to the rejection, Applicants have amended the specification and the abstract and believe that these objections are now moot. Accordingly, Applicants request that the Examiner reconsider and withdraw these objections.

Rejections Under 35 U.S.C. § 101

The Examiner has rejected claims 1, 4, 7-13, and 21-25 under 35 U.S.C. § 101 for allegedly claiming "non-statutory subject mater." Office Action, page 4. The Examiner has also rejected

claim 16 under this statute for allegedly not being supported by "either a specific and substantial and credible utility or a well established utility." *Id.* Solely to expedite prosecution and not in acquiescence to the rejection, Applicants have amended claims 1 and 16 and believe that these rejections are now moot. Accordingly, Applicants request that the Examiner reconsider and withdraw the rejections.

Rejections Under 35 U.S.C. §112, 1st Paragraph

The Examiner has rejected claim 16 under 35 U.S.C. § 112, first paragraph for allegedly not being "supported by either a specific and substantial and credible utility or a well established utility. . . ." Office Action, pages 5-6. Solely to expedite prosecution and not in acquiescence to the rejection, Applicants have amended claim 16 and believe that this rejection is now moot. Accordingly, Applicants request that the Examiner reconsider and withdraw the rejection.

Enablement Rejections Under 35 U.S.C. §112, 1st Paragraph

Claims 1, 4, 7-13, 16 and 21-25 are rejected under 35 U.S.C. § 112, first paragraph, for allegedly failing to comply with the enablement requirement. Office Action, pages 6-9. Applicants respectfully traverse the rejection.

Applicants' specification does correlate interference with invasion of *Babesia bovis* into erythrocytes with SEQ ID NO:2 and immunogenic fragments thereof. This correlation is shown in Applicants' examples in section 1.2.4 on pages 35-36 and in Example III on page 39. See also corresponding figures 5 and 13, and section 1.1.5 on pages 29-30. These examples describe an assay showing that antisera raised against SEQ ID NO: 2 and immunogenic fragments thereof inhibit invasion of *Babesia bovis* into erythrocytes.

Moreover, such an *in vitro* assay serves as a model for how SEQ ID NO: 2 or its immunogenic fragments will behave *in vivo* in animals. It is clear from Applicants' specification that in animals, *Babesia bovis* infects erythrocytes. *See* page 1, lines 24-33; and page 2, lines 18-23. This is also reflected in Gaffar, F. R. *et al.*, *Infection and Immunity* 72(5):2947-2955 (May 2004) (previously provided in an Information Disclosure Statement filed on March 14, 2006).

The first paragraph of Gaffar *et al.* describes the mechanism by which *Babesia bovis* infects animals:

> *Babesia bovis* is an obligatory intraerythrocytic bovine parasite that belongs to the phylum Apicomplexa. . . . When an extracellular merozoite enters an erythrocyte, it forms an initial reversible attachment, which leads to the reorientation of the merozoite that brings the anterior apical pole in contact with the plasma membrane of the erythrocyte [citations omitted]. A tight junction is formed, through which the parasite invades the red blood cell.

Accordingly, the *in vitro* cellular assay used by Applicants evaluating the ability of a substance to partially or fully inhibit the invasion of *Babesia bovis* into erythrocytes serves as a model for how the substance is expected to behave *in vivo* in animals. This is indeed also reflected in Franssen, F. F. J. *et al., Microbes and Infection 5*: 365-372 (2003) (provided herewith as Exhibit A) which describes the assay used by Applicants. In the paragraph spanning page 365-366, the authors introduce this invasion assay as a new *in vitro* model that mimics as closely as possible the natural invasion mechanisms of *Babesia bovis*. Hence, Applicants' cellular *in vitro* invasion inhibition assays serves as a reliable model for what biological activity or response should be expected *in vivo* in animals.

The skilled artisan upon reading Applicants' specification readily appreciates that in an animal, SEQ ID NO: 2 or its immunogenic fragments will elicit an immune response resulting in antiserum to these antigens; and that such antiserum would serve to inhibit the invasion of *Babesia bovis* into the animal's erythrocytes. The M.P.E.P. provides guidance to Examiners that challenge data in an animal model is not required where a model that demonstrates efficacy is accepted:

> The issue of "correlation" is related to the issue of the presence or absence of working examples. "Correlation" as used herein refers to the relationship between *in vitro* or *in vivo* animal model assays and a disclosed or a claimed method of use. An *in vitro* or *in vivo* animal model example in the specification, in effect, constitutes a "working example" if that example "correlates" with a disclosed or claimed method invention. If there is no correlation, then the examples do not constitute "working examples." In this regard, the issue of "correlation" is also dependent on the state of the prior art. In other words, if the

> art is such that a particular model is recognized as correlating to a specific condition, then it should be accepted as correlating unless the examiner has evidence that the model does not correlate. Even with such evidence, the examiner must weigh the evidence for and against correlation and decide whether one skilled in the art would accept the model as reasonably correlating to the condition. *In re Brana*, 51 F.3d 1560, 1566, 34 USPQ2d 1436, 1441 (Fed. Cir. 1995) (reversing the PTO decision based on finding that *in vitro* data did not support *in vivo* applications).
>
> Since the initial burden is on the examiner to give reasons for the lack of enablement, the examiner must also give reasons for a conclusion of lack of correlation for an *in vitro* or *in vivo* animal model example. A rigorous or an invariable exact correlation is not required, as stated in *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 USPQ 739, 747 (Fed. Cir. 1985):
>
> > [B]ased upon the relevant evidence as a whole, there is a reasonable correlation between the disclosed *in vitro* utility and an *in vivo* activity, and therefore a rigorous correlation is not necessary where the disclosure of pharmacological activity is reasonable based upon the probative evidence. (Citations omitted.)

M.P.E.P. §2164.02 "CORRELATION: *IN VITRO/IN VIVO*" 8th ed., revised September 2007. By using the *in vitro* invasion inhibition assay (an *in vitro* model accepted for evaluating *in vivo* behavior), Applicants have shown a reasonable correlation between the claimed peptides and their ability to interfere with invasion of *Babesia bovis* into erythrocytes. Hence, Applicants' specification enables the claims.

Solely to expedite prosecution and not in acquiescence to the rejection, Applicants have amended claim 1 to clarify that the antiserum elicited by the claimed peptides interferes with invasion of *Babesia bovis* into erythrocytes. Although recitation of "inhibits" has been replaced by "interferes," Applicants respectfully assert that the term "inhibits" need not necessarily constitute 100% inhibition. Indeed, Applicants' specification refers to partial inhibition in section 1.2.4 at page 36, lines 3-6.

Accordingly, Applicants respectfully request that the Examiner reconsider and withdraw this aspect of the rejection.

Indefiniteness Rejections Under 35 U.S.C. §112, 2nd Paragraph

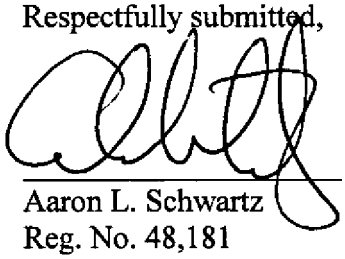
Claims 1, 4, 7-13, 16 and 21-25 are rejected under 35 U.S.C. § 112, second paragraph, for allegedly "being indefinite for failing to particularly point out and distinctly claim the subject matter which applicant regards as the invention." Office Action, pages 9-10. Solely to expedite prosecution and not in acquiescence to the rejection, Applicants have amended claims 1, 9 and 10 and believe that this rejection is now moot. Accordingly, Applicants request that the Examiner reconsider and withdraw the rejection.

CONCLUSION

Applicants do not believe that any other fee is due in connection with this filing. If, however, Applicants do owe any such fee(s), the Commissioner is hereby authorized to charge the fee(s) to Deposit Account No. **02-2334**. In addition, if there is ever any other fee deficiency or overpayment under 37 C.F.R. §1.16 or 1.17 in connection with this patent application, the Commissioner is hereby authorized to charge such deficiency or overpayment to Deposit Account No. **02-2334**.

Applicants submit that this application is in condition for allowance, and request that it be allowed. The Examiner is requested to call the Undersigned if any issues arise that can be addressed over the phone to expedite examination of this application.

Respectfully submitted,



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ABSTRACT

The invention relates to a Piroplasmid protein or an immunogenic fragment of ~~said~~ the protein, and to a nucleic acid encoding ~~said~~ the Piroplasmid protein or ~~said~~ the immunogenic fragment. Furthermore, the invention relates to cDNA fragments, recombinant DNA molecules and live recombinant carriers comprising ~~said~~ the nucleic acid. Also the invention relates to host cells comprising ~~said~~ the cDNA fragments, recombinant DNA molecules and live recombinant carriers. Finally, the invention relates to vaccines comprising a Piroplasmid protein or an immunogenic fragment of ~~said~~ the protein, to methods for the preparation of such vaccines, to the use of such proteins or fragments for vaccine purposes, and to diagnostic tests.

EXHIBIT A

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Original article

Characterisation of erythrocyte invasion by *Babesia bovis* merozoites efficiently released from their host cell after high-voltage pulsing

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Abstract

Apicomplexa are a phylum of obligate intracellular parasites critically dependent on invasion of a host cell. An in vitro assay for erythrocyte invasion by *Babesia bovis* was established, employing free merozoites obtained after the application of high-voltage to the parasitised erythrocytes. The invasion proceeds efficiently in phosphate-buffered saline solution without the requirement for any serum or medium components. The kinetics of invasion can be measured over a time span of 5–60 min after which invasion is completed at an average efficiency of 41%. The fast kinetics and high efficiency exceed those of most previously established apicomplexan invasion assays. The manipulation of intracellular calcium concentration inhibits invasion. Preincubation of merozoites at 37 °C also reduces invasion, possibly by the premature secretion of protein. Proteins that are shed into the environment during invasion were directly detectable by protein staining after 2-D gel electrophoresis. The limitations posed by the immunological detection of proteins released during in vitro invasion by other apicomplexan parasites can, therefore, be avoided by this method. A unique feature of the assay is the reversible uncoupling of invasion and intracellular development, the latter taking place only under serum-rich medium conditions. In addition, host cell attachment is uncoupled from invasion by cytochalasin B.

Keywords: *Babesia bovis*; Erythrocyte; Invasion; *Apicomplexa*; Protein secretion

1. Introduction

Babesia bovis is an apicomplexan parasite of cattle causing major damage to the livestock industry in tropical and sub-tropical areas and is transmitted by ticks of the genus *Boophilus*. Unlike malaria sporozoites, which invade hepatocytes, the sporozoites of *B. bovis* directly invade erythrocytes upon which an asexual erythrocytic cycle of duplication, erythrocyte rupture and merozoite reinvasion is initiated. Like the plasmodial erythrocytic cycle, this can cause severe pathology, which can ultimately lead to the death of the host. One clinical feature is cerebral babesiosis, characterised by clogging of the cerebral microvasculature with infected erythrocytes and associated inflammatory reactions. At least superficially, this resembles human cerebral malaria caused by *Plasmodium falciparum* [1].

The host cell invasion by apicomplexan merozoites is a conserved and critical step that is amenable to intervention by the immune system. For *Plasmodium* species and *Toxoplasma gondii*, the molecular mechanism of invasion has been investigated in some detail [2–5]. Rhoptries and micronemes, characteristic apicomplexan secretory organelles containing molecules essential to host cell invasion, harbour a complex mixture of proteins, but for *B. bovis*, only the rhoptry protein RAP-1 has been identified and studied in detail [6–8]. In addition, a function for the merozoite surface proteins MSA-1 [8] and MSA-2 [9,10] has been implied in *B. bovis* invasion.

In vitro invasion of host cells by other apicomplexan parasites is accompanied by the rapid release of parasitic proteins into the environment [4,11]. A function in the invasion process is being assigned to an increasing number of these proteins. Studies on erythrocyte invasion by most *Plasmodium* species are hampered by the low yields of viable free merozoites and the long incubation times (up to 48 h) required for quantification. This is in marked contrast with the rapid, within minutes, invasion observed in vivo. Our aim was to establish an in vitro *B. bovis* invasion assay that is

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rapidly quantifiable and efficient in numbers of invading merozoites. A novel method of merozoite release, followed by erythrocyte invasion in a protein-free buffer, meets these criteria and allows direct detection of proteins secreted during invasion, in sufficient quantities for further characterisation and sequence identification. It should help to close the gap in molecular knowledge as compared to other related parasites and provide another apicomplexan model system for studying erythrocyte invasion.

2. Materials and methods

2.1. *In vitro culture of* B. bovis

B. bovis clonal line C61411, derived from an Israeli isolate [12], was cultured in vitro according to the MASP method [13] with slight modifications. Briefly, parasites were cultured in a settled layer of bovine erythrocytes at 5% packed cell volume (PCV) in M199 supplemented with 40% adult bovine serum and 26 mM sodium bicarbonate (M199S) under an atmosphere of 5% CO_2 in air at 37 °C. Culture volumes were 1200 µl in 24-well plates, 15 ml in 25-cm^2 flasks and 50 ml in 75-cm^2 flasks. Cultures were maintained at a parasitaemia between 1% and 5% by daily dilution. For invasion studies, cultures were harvested in log phase at 8–12% parasitaemia.

2.2. *Preparation of bovine serum and erythrocytes and* B. bovis *immune serum*

Blood retrieved from a selected Friesian-Holstein heifer was defibrinated by shaking for 10 min with glass beads and centrifuged for 30 min at 4 °C at 2000 *g* for pelleting erythrocytes. Serum was centrifuged again, frozen at –20 °C and stored until use. Erythrocytes were washed four times in VyMs buffer [14], kept at 4 °C and used for maximally 14 d. Immune serum was obtained from a cow that was infected three times at 3-month intervals with *B. bovis* clonal line C61411.

2.3. *In vitro invasion assay*

B. bovis culture was centrifuged (2000 *g*, 10 min, 15 °C), after which the pellet was resuspended in an equal volume of cytomix (120 mM KCl, 0.15 mM $CaCl_2$, 2 mM EGTA, 5 mM $MgCl_2$, 10 mM $KHPO_4/KH_2PO_4$ pH 8.0, 25 mM HEPES pH 8.0). Samples of 750 µl were subjected to five intermittent (10 s, 0 °C) high-voltage pulses (2.5 kV, 200 Ω, 25 µF) in a BioRad Gene Pulser with pulse controller using 4-mm Bio-Rad cuvettes (165-2088). For the removal of soluble extracellular components from the liberated merozoites and erythrocyte ghosts, 120-µl samples were washed one to three times by resuspension in 1 ml of buffer (as indicated in the text and legends), followed by centrifugation (2000 *g*, 2 min, 15 °C). After the last centrifugation, the pellet was resuspended to a total volume of 120 µl, which was subsequently added to 1080 µl of pre-conditioned bovine erythrocytes (in 24-well plates) and transferred to an atmosphere of 5% CO_2 in air at 37 °C to initiate invasion. Pre-conditioned erythrocytes consisted of 50 µl (PCV) bovine erythrocytes (washed in PBS for three times in addition to the four washes in VyMs buffer) suspended in 1030 µl of buffer (buffer indicated in the text) and incubated under an atmosphere of 5% CO_2 in air at 37 °C for 30 min. Invasion was measured at the indicated time points by counting the parasitised erythrocytes out of a total of 5000 erythrocytes in Giemsa-stained smears. Invasion efficiency was calculated relatively to a control invasion performed in triplicate in M199S. For characterisation of the invasion process, several chemicals were added to the pre-conditioned erythrocytes and/or preincubations of liberated merozoites were performed as indicated in the text and legends. These included BAPTA-AM (20 mM stock solution in DMSO, Sigma), thapsigargin (1 mM stock in DMSO, Sigma), cytochalasin B (400 µM stock in DMSO, Sigma) and ionomycin (400 µM stock in DMSO, Sigma).

2.4. *Viability determination of merozoites*

Liberated merozoite suspension was added to an equal volume of 20 µg/ml 6-carboxyfluorescein diacetate (CFDA, Sigma) and incubated for 30 min at 20 °C in a dark place. Merozoites were resuspended in PBS after centrifugation (2000 *g*, 2 min) and examined under UV.

2.5. *Sample preparation and isoelectric focusing*

The overlaying buffer of the invasion assay incubations was collected and centrifuged twice (10 min at 2000 *g* and subsequently for 15 min 12,000 g) for the removal of all cells. The final supernatant was concentrated and desalted over 3-kDa filters (YM-3, Millipore), precipitated in a final concentration of 10% TCA (dissolved in acetone) and dissolved in rehydration solution (7 M urea, 2 M thiourea, 4% CHAPS, 2% carrier ampholyte mixture pH 3–10NL (IPG buffer) and 20 mM DTT). IEF instrumentation, IPG gels and related reagents were from APharmacia Biotech, unless otherwise indicated. Protein (50 µg) with protease inhibitor (Complete, Roche) was loaded on 13-cm IPG strips (pH 3–10NL), rehydrated (10–14 h) and focused overnight (14–17 h) in an automated run (1 min 300 V, 90 min during which voltage rises to 3500 V followed by continued focusing at 3500 V for a total of 35-40 KVh, IPGPhor™).

2.6. *Second-dimensional electrophoresis (2-DE)*

The strips were incubated in 10 ml of equilibration buffer (50 mM Tris, 6 M urea, 2% SDS, 30% glycerol, pH 8.8) containing 30 mM DTT for the first 15 min and replaced by equilibration buffer with 2.5% iodoacetamide for another 15 min. The second-dimensional SDS gel was carried out in a Hoefer SE600 system. Silver staining was used to visualise proteins after 2-DE. The images of the gels were acquired using LabScan v3.0 software on a Umax flatbed scanner and

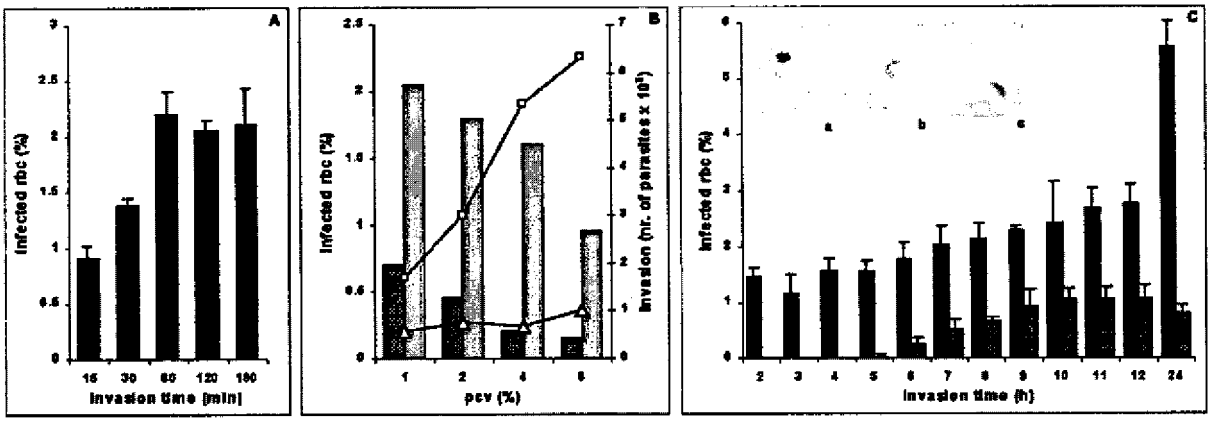

Fig. 1. (A) Percentage of invasion plotted against time. (B) Effect of PCV on invasion. Dotted bars represent percentage of invasion after 10 min, grey bars after 1 h. Lines indicate invasion in absolute numbers of parasites after 10 min (triangles) and 1 h (squares). (C) Development of parasitaemia after invasion. Black bars indicate total percentage of invaded erythrocytes. Dotted bars indicate the percentage of invaded erythrocytes harbouring a duplicated, double-pear-shaped merozoite. Error bars indicate S.D. of triplicates. Invasion performed in M199S in all panels.

later analysed with Image Master 2D v3.01 software (APharmacia Biotech).

2.7. SDS-polyacrylamide electrophoresis and Western blotting

Sample proteins were separated on a 10% SDS-PAGE gel and transferred to a nitrocellulose membrane, which was blocked with 5% skimmed milk powder in PBS, pH 7.2 for 1 h at 37 °C. All washes after each incubation step were done three times for 5 min with PBS/0.1% gelatine/0.05% Tween (PBS-GT). The blots were incubated overnight at 22–24 °C in immune sera diluted 1/500 or 1/1000 in PBS with 5% skimmed milk. After washing, the strips were incubated with anti-bovine total IgG coupled to alkaline phosphatase (diluted 1/30,000 in PBS-GT) for 1 h at 22–24 °C. The colorimetric reaction was carried out for 10–15 min with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate diluted in 100 mM Tris/100 mM NaCl/5 mM $MgCl_2$, pH 9.5.

3. Results

3.1. Kinetics of erythrocyte invasion by merozoites liberated by high-voltage pulses

Free merozoites were obtained by the application of five subsequent 2500 V pulses to *B. bovis*-infected erythrocytes (8–12% parasitaemia) that were resuspended at 50% PCV. Microscopic examination of Giemsa-stained smears showed that 99.5% of the erythrocytes were lysed. The infected erythrocytes are hardly ever encountered, but a substantial number of merozoites (<10%) reside in erythrocyte ghosts. At least 80% of the liberated merozoites were viable as defined by their ability to metabolise CFDA.

The ability to invade host cells was examined by incubation of liberated merozoites with erythrocytes (5% PCV) under standard in vitro culture conditions. The ratio of free merozoites to erythrocytes was kept identical to the ratio before liberation (e.g. 8–12%). Fig. 1A shows that invasion proceeds rapidly (0.91% parasitaemia at 15 min), reaching a plateau at 2.20% after 60 min. Invasion efficiency can be defined as the observed parasitaemia after reinvasion divided by the maximal number of potentially invasion-competent parasites (2 × percentage of erythrocytes harbouring a duplicated parasite in the culture from which the liberated parasites were obtained). Six independent invasion experiments performed over a time span of several months yielded a mean invasion efficiency of 41.6 ± 15.6% (standard deviation, S.D.), reaching 0.8–3.8% absolute parasitaemia, after 60 min. Careful examination and focusing under the light microscope showed that many parasites were already in contact with erythrocytes (juxtaposed to, but often also looking as if being located on the top of, an erythrocyte) after 15 min. The sum of parasites contacting and having invaded an erythrocyte after 15 min approximately equals (2.54 ± 0.14%) the number of parasites that have actually invaded after 60 min. Very few parasites contacting an erythrocyte were left after 60 min, whereas the parasites that did not invade lay free or clumped together. Fig. 1B displays the effect of erythrocyte PCV on invasion. Relative parasitaemia becomes highest at a PCV of 1%, but the absolute number of invaded merozoites is highest at a PCV of 8%, both after 10 min and 1 h.

B. bovis infections as well as in vitro cultures are asynchronous, and methods for synchronisation are lacking. The span of a life cycle has only been estimated from the average multiplication rate. In the current invasion assay, it can be assumed that immature merozoites, liberated by high-voltage, do not develop any further, whereas only mature merozoites are invasion competent, thus providing a synchronised start. This does not result in synchronous growth (Fig. 1C). Upon the appearance of the first duplicated double-pear-shaped parasites after 5 h, the onset of a gradual increase in parasitaemia is observed. This defines the minimal time span of a complete life cycle at ~6 h, but a sudden

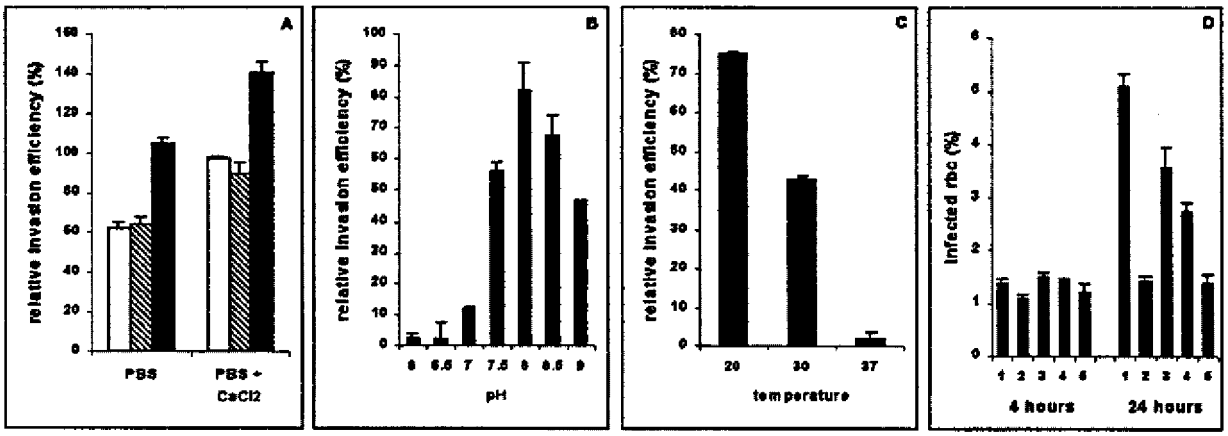

Fig. 2. (A) Effect of medium composition on invasion. Merozoites were washed once in PBS (striped bars) or in PBS + 1 mM $CaCl_2$ (black bars) or were not washed (grey bars) after lysis by high-voltage. Immediately after washing, merozoites were allowed to invade erythrocytes for 1 h in PBS pH 8.0 or in PBS pH 8.0 + 1 mM $CaCl_2$ as indicated. Invasion was plotted relative to invasion performed in M199S (control performed in triplicate), which was taken as 100%. (B) Effect of pH on invasion. Invasion was performed for 30 min in PBS that was adjusted to the indicated pH and plotted relatively against invasion performed in M199S. (C) Effect of preincubation of merozoites on invasion. Merozoites were incubated for 30 min in PBS pH 8.0 prior to invasion at the indicated temperature, after which invasion was performed for 30 min in PBS pH 8.0. Efficiency was plotted against invasion in PBS pH 8.0 by merozoites that were not preincubated. (D) Development of invaded parasites after the addition of growth medium. Invasion was performed for 2 h in M199S (bar 1) or in PBS pH 8.0 (bars 2-5). M199S or PBS pH 8.0 was either not replaced (bars 1 and 2) or replaced by M199S after 2 (bar 3), 4 (bar 4) or 24 h (bar 5). Infected erythrocytes were counted after 4 and 24 h as indicated. Replacement of PBS after 24 h (bar 5) showed abnormally developed parasites at that time point, but within 72 h normal growth rate is restored (not shown). All assays were performed in triplicate and error bars indicate S.D.

burst of duplicated and subsequently reinvaded parasites is not observed, indicating a wide spread in lifecycle duration between individual parasites. In Fig. 1C, a 3.8-fold multiplication is reached after 24 h, which is within the range (3.5- to 5-fold in 24 h) that is observed in a continuously growing culture.

3.2. Effects of medium composition and merozoite pre-treatment on erythrocyte invasion

Dependence of *B. bovis* in vitro growth on a rich culture medium supplemented with 40% adult bovine serum (M199S) complicates dissection of the components involved in invasion, intra-cellular establishment and growth. Fig. 2A shows that invasion can take place in the absence of serum and medium. A 35% reduction in invasion efficiency was observed when performed in PBS instead of M199S (bar 1). Addition of 1 mM $CaCl_2$ restored relative invasion efficiency to 96% (bar 4). Washing of merozoites in PBS prior to invasion had no significant effect on efficiency (bars 2 and 5), whereas washing in the presence of 1 mM $CaCl_2$ enhanced invasion by ~40% (bar 3 and 6). Repeated washing caused a gradual decline in efficiency, but a threefold wash and subsequent invasion in PBS/$CaCl_2$ still permitted comparable invasion levels as observed with non-washed merozoites in M199S (not shown). A pH from 7.5 to 9.0 was compatible with invasion, with an optimum at pH 8.0 (Fig. 2B). A sharp drop in invasion efficiency was seen at pH 7.0. Invasion was abolished at 32 or 42 °C (not shown). Preincubation of merozoites for 5 min at 37 °C completely prevented invasion, although no merozoite agglutination or changes in shape were observed by microscopy. Thirty-minute preincubation of merozoites at 20 or 30 °C was much better tolerated than 37 °C (Fig. 2C).

Light microscopy of Giemsa-stained smears showed invaded parasites (either in PBS or in M199S) as condensed (pycnotic) intra-erythrocytic particles (Fig. 1C, insert a). In M199S, parasites rapidly (2–3 h) develop into trophozoite forms with a clear blue cytoplasm (Fig. 1C, insert b), but after performing invasion in PBS, any further development is blocked and parasites remain pycnotic. Normal development is rapidly restored when PBS is replaced by M199S after 2 or 4 h (Fig. 2D). Addition of M199S after 24 h restored parasite growth only after a 24 to 72-h lag phase (not shown).

3.3. Effects on invasion by inhibitors affecting cytosolic Ca^{2+} concentration or actin polymerisation

Cytochalasins prevent actin polymerisation by capping the barbed end of actin [15], and their inhibitory effect on host cell invasion of several apicomplexan parasites suggested the involvement of an actin/myosin motor system [16–18]. *B. bovis* invasion is completely inhibited by 5 μM cytochalasin B (Fig. 3A) and examination of Giemsa-stained preparations identified many merozoites contacting an erythrocyte. Of these, many are spread out on the stained preparation as if the tip of the merozoite is contacting the erythrocyte (Fig. 1C, insert c).

Extracellular Ca^{2+} (1 mM) had a small positive effect on invasion by *B. bovis* (Fig. 2A). For examination of the effect of cytoplasmic free Ca^{2+} on invasion, merozoites were preincubated with ionomycin (a Ca^{2+} ionophore giving rise to increased cytosolic Ca^{2+} by influx from the extracellular millieu or intracellular Ca^{2+} stores), BAPTA-AM (a specific

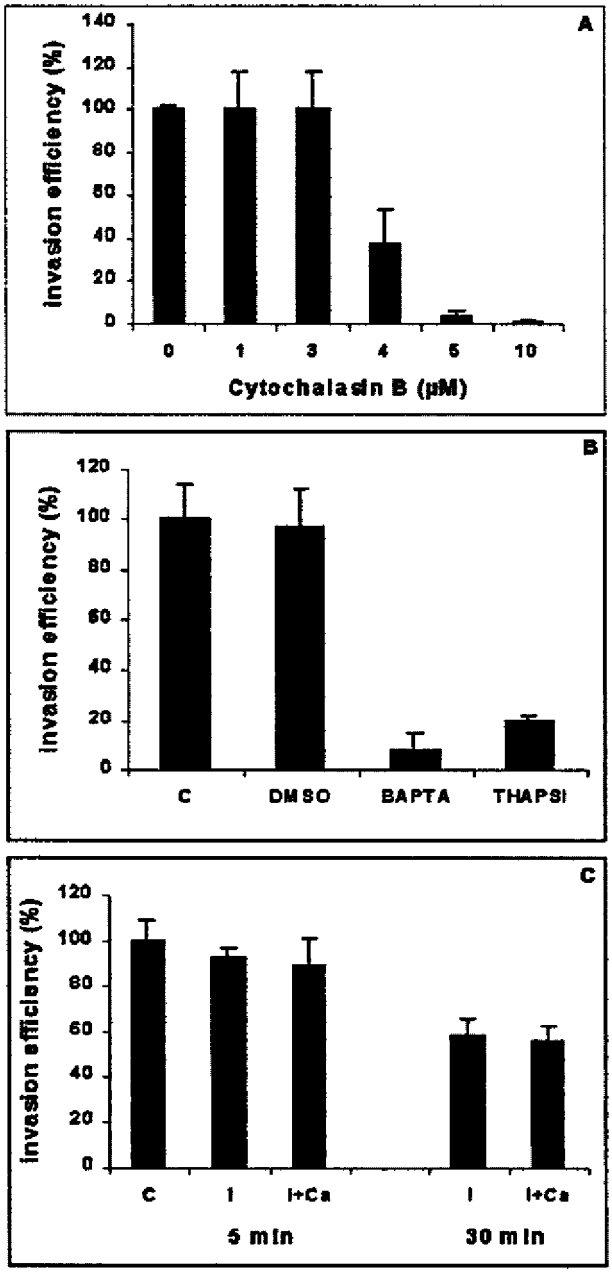

Fig. 3. (A) Inhibition of invasion by cytochalasin B. Invasion was performed in M199S after 15 min preincubation of merozoites with cytochalasin B at the indicated concentration and determined after 1 h. (B) Effect of BAPTA-AM and thapsigargin on invasion. Invasion was performed for 1 h in M199S without preincubation (C) or after preincubation for 90 min at 20 °C in PBS + 0.5% DMSO, 90 min preincubation at 20 °C with 100 µM BAPTA-AM or 30 min preincubation at 20 °C with 1 µM thapsigargin. (C) Effect of ionomycin on invasion. Invasion was performed for 1 h in M199S without preincubation (C) or after preincubation for 5 and 30 min, respectively, with ionomycin in PBS pH 8.0 or PBS pH 8.0 + 1 mM $CaCl_2$. Error bars indicate S.D. of triplicates.

chelator of cytosolic Ca^{2+}) or thapsigargin (an inhibitor of sarco/endoplasmic reticulum Ca^{2+}-ATPases that transport cytosolic Ca^{2+} back into the sarco/endoplasmic reticulum

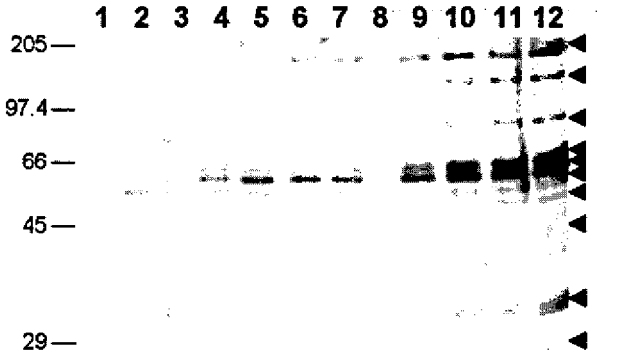

Fig. 4. Western blotting of proteins released during invasion of erythrocytes. Lanes 3–7, control incubations for determination of proteins released by free merozoites in the absence of erythrocytes. Lanes 8–12, invasion in PBS pH 8.0. Released proteins were harvested after 0, 5, 15, 30 and 60 min (lanes 3 and 8, 4 and 9, 5 and 10, 6 and 11, 7 and 12, respectively). Lane 2 is a control with non-infected erythrocytes used for invasion for 1 h. Lanes 2–12 were incubated with immune serum; lane 1 is identical to lane 12 but incubated with preimmune serum.

[19]). Fig. 3B shows that preincubation with 100 µM BAPTA-AM reduces invasion more than 10-fold, whereas 1 µM thapsigargin reduces invasion fivefold. Ionomycin had a moderate effect in the presence or absence of extracellular Ca^{2+}, resulting in 40% inhibition after 30-min preincubation (Fig. 3C). In all cases the viability of merozoites was not affected by the inhibitor treatment, as indicated by their capacity to metabolise CFDA.

3.4. Invasion is accompanied by the rapid release of parasite specific antigens into the surroundings

B. bovis proteins shed at different time points during invasion were separated by SDS-PAGE, blotted and were labelled with *B. bovis* antiserum. Antigenic proteins of 60 and 64 kDa are released within 5 min upon transfer of the incubation mixture from 0 to 37 °C (Fig. 4). The same bands are also observed in a control experiment in which no erythrocytes were added to the liberated merozoites (Fig. 4, compare lanes 3 and 8 with 4 and 9). During 60 min of invasion, shedding of antigenic proteins was continued, resulting in the detection of additional antigenic bands with a gradually increasing intensity over time (lanes 9–12). In a control experiment where invasion is prohibited by the absence of erythrocytes, no gradual increase in the intensity and number of bands was observed (lanes 5–7).

Most of the protein in the samples is still derived from erythrocyte ghosts in the form of cytoskeletal proteins and associated haemoglobin that could not be removed by repeated washes at 0 °C but partly became solubilised during the incubation at 37 °C. This limits the amount of *B. bovis*-derived proteins that can be loaded on an SDS-polyacrylamide gel and, in combination with the limited resolution obtained by 1D-SDS-PAGE, restricts the identification of secreted/shedded proteins by direct protein stain-

Fig. 5. 2-D gel electrophoresis (12.5%) of proteins released during invasion. Panel A, supernatant of invasion of erythrocytes with liberated merozoites. Panel B, supernatant of "invasion" with high-voltage lysed extract of mock-infected erythrocytes. Invasion was performed for 1 h in PBS pH 8.0 in a volume of 15 ml. Merozoites were washed in PBS pH 8.0 for three times prior to invasion. Parasitaemia after invasion was determined at 0.84%. The arrows indicate prominent spots that cannot be detected in control of panel B. The boxed area contains many "invasion specific" spots.

ing. Employing the capacity and resolving power of 2-D-protein gel electrophoresis, a large number of *B. bovis*-specific major and minor protein spots could be detected after electrophoresis of in vitro invasion supernatants (Fig. 5). Panel A shows the proteins present in 2 ml of supernatant derived from an experiment in which merozoites were allowed to reinvade for 1 h. Panel B shows a control experiment in which uninfected erythrocytes were lysed by high-voltage and further washed and used for invasion under identical conditions as in panel A. Thus, panel B shows all the erythrocyte proteins (mainly monomeric and multimeric haemoglobin B and cytoskeletal proteins) that were not solubilised during the washing steps but only after incubation for 1 h at 37 °C (no resemblance can be detected with a proteome map of cytosolic bovine erythrocyte proteins available at http://www.expasy.ch/ch2dothergifs/publi/rbc.gif, indicating that no significant lysis of erythrocytes takes place during invasion). Panel A shows in addition a large amount of spots that represent merozoite proteins released into the supernatant during 1 h of invasion. Merozoite-derived proteins were particularly abundant in the boxed region of panel A. The arrows mark other prominent spots that are not present in the control. At least 110 spots are present in panel A that could not be detected in panel B (some of the fainter spots are close to the detection limit and may thus just have been missed in panel B).

4. Discussion

Studies on *B. bovis* erythrocyte invasion may help in pinning down conserved [20,21] and species-specific aspects of the molecular mechanisms of apicomplexan host cell invasion. Here we have presented an invasion assay, based on the liberation of *B. bovis* merozoites by high-voltage pulses, with the following hallmarks. (i) ~40% of the liberated "invasion-competent" merozoites invade an erythrocyte within 1 h. (ii) Invasion proceeds efficiently in PBS, thereby providing an excellent model system for studying the effects of exogenously added components without having to take into account the effects of the many undefined substances present in serum-rich media. (iii) During invasion, proteins are released that can be concentrated without using selective procedures like immunoprecipitation that otherwise have to be used to remove the excessive amounts of protein present in serum-rich media. (iv) The assay uncouples invasion and intracellular development, enabling the systematic reconstitution of medium components required for intracellular growth.

These features allow more accurate and specific in vitro studies of *B. bovis* erythrocyte invasion than currently is the case for most *Plasmodium* species. Light microscopy indicates that within 5 min most parasites that will invade within the next hour have become attached to an erythrocyte, whereas 41% of the maximum level of invasion is already reached after 15 min. A qualitative description of erythrocyte invasion by *P. knowlesi* has shown that invasion can be accomplished within minutes after initial contact [22], but in contrast, invasion experiments by other *Plasmodium* species are typically performed in a time span ranging from 4 [23] to 48 h [24].

Studies on apicomplexans have shown that host cell receptors are rapidly secreted from micronemes upon initial cell-cell contact, followed by the secretion of additional proteins from rhoptries and release of plasma membrane-bound proteins [4,25]. The results obtained for *B. bovis* fit

into such a model. Immune-recognised proteins of 60 and 64 kDa are secreted upon transfer to 37 °C followed by an accumulation of additional bands in the next hour of invasion. Immediate secretion of 60 and 64 kDa proteins is also seen upon transfer to 37 °C in the absence of invasion (control with no erythrocytes added). This may be a premature secretion and could explain the reduced invasion that is seen after a brief preincubation of merozoites at 37 °C. As preincubation at 20 °C has no detrimental effect on invasion and does not lead to protein release, the rapid release of 60 and 64 kDa proteins could result from an enzymatically regulated event. This may have been triggered by the interaction with host cell receptors that might also be present in the control in the form of erythrocyte ghost membranes.

Invasion in medium containing 40% bovine serum is fully productive, as is shown by the subsequent growth rate. In the first 24 h, this lies within the 3.5- to 5-fold multiplication range of a continuous growing culture. Invasion performed in PBS followed by a change to standard growth medium after 1 h is equally productive, from which can be concluded that the merozoite carries all the requirements for optimal invasion. A large variation in intra-erythrocytic life cycle duration was determined with a minimal length of 6 h. After 11 h, parasitaemia has increased from 1.55% (x) to 2.7% (y) with 1.7% (z) of the erythrocytes harbouring a single trophozoite. Thus 35% ($(z-(y-x))/x$) of the parasites have not duplicated yet, indicating that the time required to develop to a duplicated double-pear-shaped meront is an important component in the asynchronous growth of *B. bovis*. This can be an inherent property of *B. bovis* development but could also be due to sub-optimal in vitro growth conditions.

Five micromoles of cytochalasin B gives 95% inhibition, whereas 3 μM has no effect. During a 48-h period of growth, 0.4 μM of cytochalasin B inhibits *B. bovis* growth by 25%, possibly indicating a stronger effect on the function of contractile microfilaments during replication [26]. The effect on invasion was not measured by this method. Sensitivity is in between the values observed for *Eimeria tenella* (82% inhibition by 10 μM) and *P. knowlesi* (~90% inhibition by 1 μM) [16,18]. The frequent observation of merozoites contacting an erythrocyte by their tip is reminiscent of observations on *P. knowlesi* and *T. gondii* parasites that were preincubated with cytochalasin [16,17]. Cytochalasins may prevent movement of the parasite into an invaginating vacuole by blocking an actin/myosin motor system [27] without blocking the attachment to the host cell after apical reorientation. Studies on the role of Ca^{2+} in apicomplexan host cell invasion [23,28–31] gave rise to a model in which protein secretion during invasion is a stimulus-coupled event with Ca^{2+} as a second messenger. Chelation of intracellular Ca^{2+} by BAPTA-AM inhibits *B. bovis* invasion but is a harsh tool that will also affect other biological processes. The strong inhibition by thapsigargin points at the involvement of the ER, as this drug inhibits repletion of this calcium store. This effect of thapsigargin has been observed for other apicomplexans too, but recently [32] it was shown that incubation with thapsigargin does not give rise to an increase in cytosolic Ca^{2+}, making the effect of this drug on invasion still puzzling. The observed stimulating effect of 1 mM extracellular Ca^{2+} could indicate the use of extracellular Ca^{2+} for a transient increase in cytosolic Ca^{2+} but it may equally well positively affect essential protein interactions between host cell and merozoite during invasion.

During continuous in vitro cultivation of *B. bovis*, a mixture of proteins is shed that has been described as culture-derived exoantigens [33–36]. Their origin and function are still largely unknown. The immunogenicity of these proteins provoked vaccination studies that have given promising results in other *Babesia* species as well. Most likely, the immunogenic proteins that are secreted during host cell invasion (Fig. 4) are part of the proteins present in exoantigens. *B. bovis* exoantigens, harvested over periods of 48 h, will contain a lot of other parasitic proteins (e.g. from decaying parasites) and are usually studied with immunological tools, as they are masked by an excessive amount of serum and host cell proteins. The *B. bovis* proteins released during in vitro invasion in PBS can be identified directly by 2-D gel electrophoresis. The number of protein spots detected by far exceeds the number of antigenically detected proteins after 1-D electrophoresis (10 in Fig. 4) and also the number of antigenically detected exoantigens [33,35,36]. The identification and initial functional characterisation of these additional proteins is now enabled by their visualisation by 2-DE. Identification of the proteins not detected by antibodies may also be relevant for vaccine development; the inherent immunogenicity of a parasite molecule does not correlate with its protective capacity [37].

In summary, the strength of the assay is its speed, efficiency and independency of medium composition. The effects of specific invasion inhibitors or conditions on the kinetics of secretion of individual proteins can be studied on a short time-scale (5 min–1 h) by the use of 2-DE in order to unravel the molecular mechanisms involved. The protein spots are amenable to identification by a proteomics approach, considering the amounts that can be produced (the assay can without difficulty be performed in 50-ml quantities). Rare events can be monitored directly, as the application of 50 ml of invasion supernatant to a 2-D gel will allow the detection of 20 molecules of 60 kDa secreted by a single merozoite (assuming the invasion of 2% of the erythrocytes and a detection limit of 1 ng protein by silver staining).

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